The regulation of vascular tetrahydrobiopterin bioavailability

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Abstract

6*R* L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) is an essential cofactor for several enzymes including phenylalanine hydroxylase and the nitric oxide synthases (NOS). Oral supplementation of BH_4 has been successfully employed to treat subsets of patients with hyperphenylalaninaemia. More recently, research efforts have focussed on understanding whether BH_4 supplementation may also be efficacious in cardiovascular disorders that are underpinned by reduced nitric oxide bioavailability. Whilst numerous preclinical and clinical studies have demonstrated a positive association between enhanced BH_4 and vascular function, the efficacy of orally administered BH_4 in human cardiovascular disease remains unclear. Furthermore, interventions that limit BH_4 bioavailability may provide benefit in diseases where nitric oxide over production contributes to pathology. This review describes the pathways involved in BH_4 bio-regulation and discusses other endogenous mechanisms that could be harnessed therapeutically to manipulate vascular BH_4 levels.

Key words:

Tetrahydrobiopterin; GTP cyclohydrolase 1; Nitric Oxide Synthase; Endothelial

Word Count: 5499

1. Introduction

6*R* L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) has, in recent years, garnered considerable attention as a therapeutic target for a number of pathologies, notably neurological and cardiovascular diseases. The ability of BH_4 to affect such a diverse range of biological systems can be attributed to its essential cofactor role for numerous enzyme families, namely the aromatic amino acid hydroxylases (AAAH) phenylalanine, tyrosine and tryptophan hydroxylase [1, 2], the nitric oxide synthases (neuronal/ NOS 1, inducible/NOS 2, endothelial/NOS3) [3, 4] and alkylglycerol mono-oxygenase (or glycerylether monooxygenase - GEMO) [5, 6]. It is also likely that there are a number of other biological roles for BH_4 which are yet to be determined [7].

The pharmacological enhancement of intracellular BH_4 levels holds therapeutic promise for a number of cardiovascular disorders underpinned by reduced nitric oxide (NO) and thus endothelial dysfunction. Conversely, attenuating the rise in intracellular BH_4 which occurs in response to proinflammatory stimulation would be of benefit in diseases such as inflammatory pain and circulatory collapse as observed in septic shock patients. This review summarises the current evidence supporting the modulation of BH_4 levels and discusses alternate strategies to regulate its bioavailability at an intracellular level.

2. BH_4 reaction mechanisms

Endogenous BH_4 levels are regulated by a number of highly conserved synthetic and recycling enzymes (summarised in Fig. 1) that ensure intracellular BH_4 levels are maintained at or below saturating levels for enzymes that require it as a cofactor [8].

These include the:

i) '*De novo* biosynthetic pathway' utilising GTP as a substrate

ii) 'Regeneration/recycling pathway' which regenerates BH_4 following its bioconversion during the reactions catalysed by AAAH and GEMO

iii) 'Salvage pathway' which can regenerate BH_4 from 7,8 dihydrobiopterin (BH_2).

The *de novo* biosynthesis of BH_4 proceeds in a Zn^{2+}, Mg^{2+} and NADPH-dependent manner from GTP via two reaction intermediates; 7,8-dihydroneopterin triphosphate and 6-pyruvoyl-tetrahydropterin. The enzymes involved in these reactions are; GTP cyclohydrolase 1 (GCH1), 6- pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR), with the latter step believed to occur in two stages with different reaction intermediates [8-11] (Fig. 1). Of these biosynthetic enzymes,

GCH1 commonly forms the rate limiting step, and thus represents a tractable target for the pharmacological manipulation of endogenous BH_4 levels.

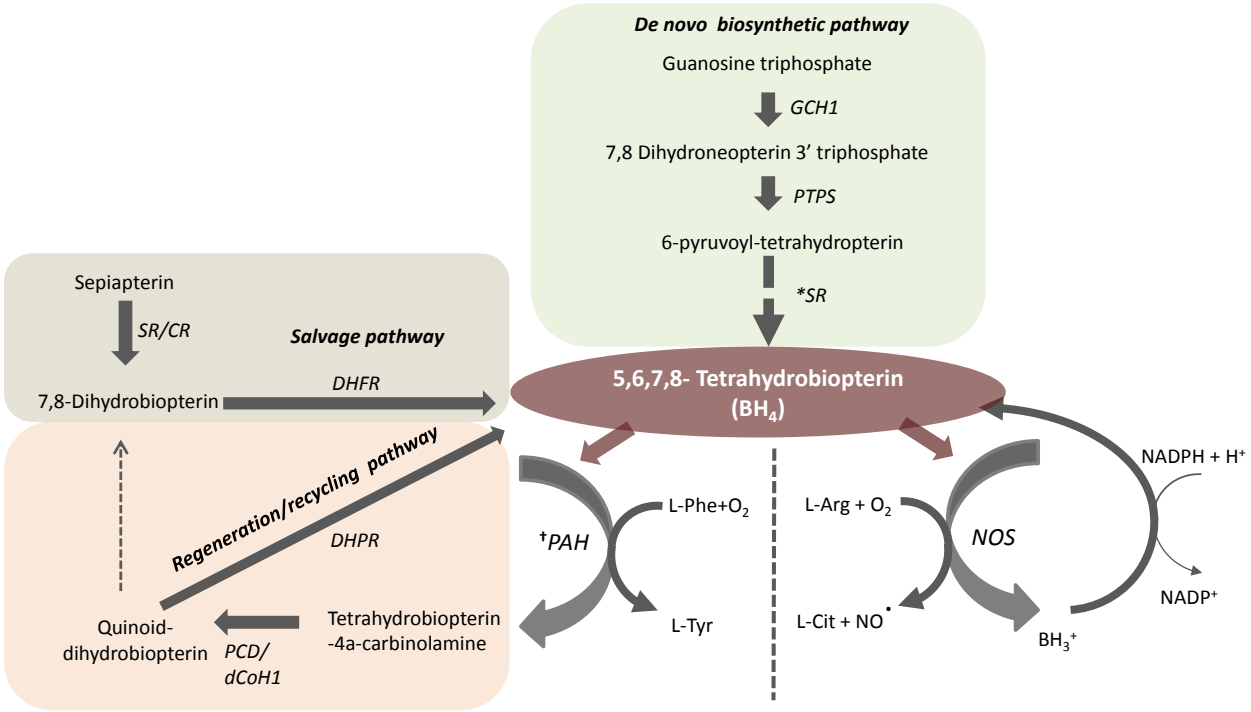

Figure 1: **BH_4 biosynthetic and regulatory pathways**. GCH1, GTP cyclohydrolase 1; PTPS, Pyruvoyl tetrahydropterin synthase; SR, sepiapterin reductase; CR , carbonyl reductase; PAH, phenylalanine hydroxylase; NOS, nitric oxide synthase; PCD/dCoH1 pterin-4a-carbinolamine dehydratase; dCoH1dimerization cofactor of HNF-1; DHPR, dihydropteridine reductase; DHFR, dihydrofolate reductase. *Sepiapterin reductase mediated step during de novo biosynthesis is believed to occur in two stages with two different reaction intermediates, but this has been excluded from the diagram for simplification. †For simplification, tyrosine hydroxylase, tryptophan hydroxylase and glyceryl ether monooxygenase have been excluded from the diagram.

Enzymes for which BH_4 serves as a cofactor, are all mixed function mono-oxygenases. Mono-oxygenases utilise molecular oxygen during the reaction, incorporating one oxygen atom into the substrate whilst the second oxygen atom is reduced to water. The reaction mechanisms of enzymes that use BH_4 as a cofactor have been summarised in extensive reviews and will not be detailed further here [8, 10]. However, it is important to note that the BH_4 turnover products and regeneration reactions differ for the different enzyme families (Fig. 1). During the reactions catalysed by PAH and other AAAH, BH_4 is initially oxidised to tetrahydrobiopterin-4a-carbinolamine and two further enzymatic steps are involved in the subsequent dehydration and reduction back to BH_4.

In contrast, it is clear that NOS uses BH_4 in a different manner. Numerous functions of BH_4 have been proposed, including structural stabilisation of the NOS dimer and eliciting conformational changes that increase L-arginine binding [12-16] . However, current opinion suggests that a key biochemical

mechanism by which BH_4 supports NOS mediated catalysis, is through electron donation to the heme iron located within the NOS oxygenase domain [17-19].

During NOS mediated catalysis, electrons originating from the NOS reductase domain, are carried to the NOS oxygenase domain, where two successive mono-oxygenase reactions occur. These reactions involve the initial oxidation of L-arginine to the intermediate N-hydroxy-L-arginine (NOHA), which is then further converted to the end products L-citrulline and nitric oxide (NO) [20]. Each of these mono-oxygenase reactions requires BH_4.

The oxygenase domain contains a ferric heme group and BH_4 has been shown to bind in close proximity to this group [21]. Electrons originating from the NOS reductase domain reduce the ferric heme to a ferrous state, enabling O_2 to bind. This leads to the formation of a ferric heme-superoxy intermediate (Fe^{III}-O_2^-). It is believed that BH_4 donates an electron at this point, thus reducing the ferric heme-superoxy species to form a heme-oxy species facilitating L-arginine oxidation. During this process, BH_4 itself becomes an enzyme bound BH_3^+ radical [18, 22-26]. In contrast to AAAH mediated catalysis, BH_4 does not undergo regeneration by external enzymes, but rather is regenerated from the protonated BH_3^+ radical by electrons supplied from the NOS reductase domain.

3. BH_4 oxidation and NOS 'uncoupling'

The pathophysiological importance of BH_4 during NOS mediated catalysis is apparent in situations where BH_4 bioavailability is low or absent (due to oxidation or insufficient biosynthesis/recycling). Under these circumstances, BH_4 is no longer available to reduce the ferric heme-superoxy species in NOS, leading to its dissociation and the release of superoxide (O_2^-) – a phenomenon termed 'NOS uncoupling'. Thus, BH_4 deficiency and eNOS uncoupling contributes to endothelial dysfunction not only by directly reducing NO production but also by increasing NO scavenging due to O_2^- formation. This potential for NOS to generate reactive oxygen species is thought to further potentiate vascular dysfunction and highlights the importance of sustaining sufficient BH_4 levels for full NOS functionality, within the intracellular pool.

BH_4 is itself highly susceptible to auto-oxidation, through its interaction with reactive oxygen species – in particular peroxynitrite ($ONOO^-$), which is formed via the interaction of NO and O_2^-. During the reaction with $ONOO^-$, BH_4 is believed to initially be converted to a BH_3^+ radical which can be further oxidised to BH_2, although this final step can be prevented by ascorbate [27].

A further potential detrimental consequence of BH_4 oxidation, is that its oxidation product, BH_2 can bind to NOS with approximately the same affinity as BH_4, but serves no cofactor function. BH_2 may

therefore compete with, or potentially displace, BH_4 binding to NOS [28]. Thus, the ratio of BH_4:BH_2 in biological samples, rather than just BH_4 alone, may be considered a more important determinant of final NOS functionality [29].

The function of the recycling enzyme dihydrofolate reductase (DHFR) in sustaining BH_4 levels is likely to become especially important in situations where BH_4 has been subject to oxidative inactivation. It is known that DHFR regenerates BH_4 from BH_2 [30] (Figure 1) and biopterins may shuttle into cells via specific transporters [31]. It remains to be fully determined whether the DHFR pathway itself becomes dysfunctional in cardiovascular disease states.

In summary, insufficiency of BH_4 (potentially as a result of oxidation or reduced biosynthesis) leads to NOS uncoupling and the subsequent generation of reactive oxygen species, which can further oxidise and limit BH_4 bioavailability. This may result in a vicious cycle of sustained NOS uncoupling, reduced NO bioavailability and enhanced oxidative stress, further potentiating vascular dysfunction. Thus, pharmacological interventions that enhance BH_4 bioavailability within endothelial cells hold significant therapeutic potential for the treatment of numerous cardiovascular disorders.

4. BH_4 and phenylketonurea (PKU)

BH_4 was first identified as a cofactor for the aromatic amino acid hydroxylases (AAAH) and was shown to be necessary for the metabolism of phenylalanine, tyrosine and tryptophan [1, 2, 32] and consequently the eventual biosynthesis of noradrenaline, dopamine and 5-hydroxytryptamine. There are a number of neurological disorders in humans that are related to abnormal BH_4 bioavailability and metabolism [33-38]. Of these, phenylketonurea (PKU) has been especially well characterised. Mutations in the phenylalanine hydroxylase (PAH) gene can lead to protein misfolding with loss of function and lead to the development of PKU. PKU is characterised by elevated circulated phenylalanine (hyperphenylalaninaemia) which can lead to impaired brain development and significant mental retardation during early life, and is the most common inborn error of amino acid metabolism in European descended populations [39]. A strict phenylalanine (L-Phe) restricted diet (often with poor patient compliance) was for many years the only strategy implemented to control circulating L-Phe levels in PKU patients.

The clinical importance of BH_4 was first highlighted following the identification of atypical variants of hyperphenylalaninaemia who displayed neurological degeneration despite phenylalanine restriction [36, 37]. Fortunately, research efforts focused on understanding whether pharmacological BH_4 supplementation could be used to treat PKU patients, led to the clinical development of sapropterin

dihydrochloride (Kuvan), a synthetic oral BH_4 supplement which has been successfully employed to increase dietary L-Phe tolerance and maintain blood L-Phe levels within an acceptable range, in a subset of hyperphenylalaninaemic patients (termed BH_4 responsive) [40-43].

5. Regulation of nitric oxide bioavailability

Nitric oxide (NO) orchestrates a variety of important biological effects throughout the body, given the widespread distribution of the three NOS isoforms. These diverse biological effects include long term potentiation and memory formation, smooth muscle vasodilatation, inhibition of platelet aggregation and host defence – forming part of the inflammatory response to a microbial infection [44-47]. Given the diverse and profound effects of NO on various target tissues, there has been a great deal of interest in pharmacologically modulating NO bioavailability.

Following the discovery of endothelium derived relaxing factor and its subsequent identification as NO [46, 48] it has become well recognised that impaired vascular NO signalling contributes to the phenomenon of 'endothelial dysfunction', a hallmark feature of numerous cardiovascular diseases [49-51]. NO supplementation using agents such as organic nitrates (e.g. glyceryl trinitrate and isosorbide mononitrate) for the treatment of coronary artery disease, or inhaled NO gas for the treatment of pulmonary hypertension in neonates, have been employed clinically for many years. Unfortunately, the prolonged use of these agents is often limited by the development of tolerance and potentially fatal rebound effects following withdrawal (inhaled NO) [52-55].

Similarly, the inhibition of NOS for the treatment of refractory hypotension in septic shock patients showed initial benefits on hemodynamic responses, but overall resulted in an increased mortality rate, indicating the complexities of non selectively inhibiting all NOS isoforms [56, 57].

The difficulties associated with regulating vascular NO through the use of direct NO donors/NOS inhibitors, naturally led to investigations of endogenous NOS regulatory mechanisms, as an alternative method to control NO bioavailability. Such investigations have primarily studied interventions that can enhance vascular NO levels for the treatment of endothelial dysfunction and include enhancing L-arginine substrate bioavailability [58, 59] and introducing antioxidants to limit the destruction of NO by reactive oxidant species [60, 61]. Unfortunately, studies investigating the chronic administration of these agents suggests that such interventions lack clear efficacy and in some cases

worsen outcome, although it should be noted that there are inherent complexities with the design of these types of clinical trial [62-66].

Another possible target for regulating NO levels is the enzyme dimethylarginine dimethylaminohydrolase (DDAH). DDAH metabolises the endogenously produced NOS inhibitors, asymmetric dimethylarginine (ADMA) and L-N[G] monomethyl-L-arginine (L-NMMA). However, to date only selective inhibitors of DDAH (which lead to an increase in ADMA) have been developed, suggesting that DDAH may be a more tractable target for diseases characterised by elevated NO such as inflammation and septic shock [67-70]. Pharmacological DDAH activators may however, be successfully developed in the future as a method to reduce endogenous ADMA/L-NMMA and enhance vascular NO bioavailability within the cardiovascular system.

6. Regulation of BH_4 and vascular function

Intensive investigation over recent years has focused on understanding whether increasing BH_4 levels may also elicit therapeutic benefits for the treatment of cardiovascular disorders underpinned by endothelial dysfunction. Numerous rodent and clinical studies investigating genetic (GCH1 gene manipulation or human polymorphisms) have shown that loss of GCH1 activity exacerbates vascular dysfunction whilst over expression is protective, as described within section 7.1. Similarly animal studies and small scale acute clinical studies using pharmacologically supplemented BH_4, have demonstrated a correlation between enhanced BH_4 levels and improved vascular function [71-99] and Tables 1 and 2. The chief limitation of these clinical studies, was the high doses of BH_4 administered 50-100 μM compared to physiological plasma levels of 10–50 nM [100] which may have elicited NOS independent or other non specific effects [96, 101, 102]. Furthermore, only the acute vascular effects of BH_4 were assessed in these clinical studies and BH_4 was typically delivered via an intracoronary/intra arterial infusion, which is unrepresentative of a suitable route of administration for chronic disease management.

Clinical trials investigating oral BH_4 supplementation have shown varied efficacy in numerous disorders with an apparent lack of efficacy in diseases such as hypertension and coronary artery disease [103-110] and Table 3. These findings may be explained by either rapid clearance of BH_4 after oral administration and/or enhanced oxidation following adsorption, with a consequent decrease in the vascular BH_4:BH_2 ratio, despite a clear elevation of BH_4 in the plasma pool [103]. Indeed, co-administration with ascorbate has been shown to enhance BH_4 bioavailability and in certain cases

improve vascular function, possibly through chemical stabilisation and increased regeneration of BH_3^+ to BH_4 [29, 106, 108, 111, 112]. However, studies in larger cohorts of patients would be required to determine whether this dual (BH_4 plus antioxidant) intervention would be efficacious on a chronic basis.

The cellular deposition of BH_4 after administration appears to be cell type dependent and either involves direct uptake of BH_4 or initial oxidation to BH_2 and subsequent reduction back to BH_4 by dihydrofolate reductase (DHFR) via the salvage pathway [30]. An emerging literature indicates that DHFR also plays an important role in the maintenance of cellular BH_4 levels and NOS functionality. Enhanced hydrogen peroxide production arising from angiotensin II treatment reduces DHFR expression, siRNA knockdown of DHFR exacerbates NOS uncoupling and DHFR over expression elicits a restorative effect [113-116]. These studies suggest that DHFR may itself represent a therapeutic target for regulating vascular BH_4 levels and NOS functionality.

Thus, whilst the evidence clearly suggests that enhancing BH_4 within endothelial cells is beneficial, these recent clinical trials suggest that there are limitations to achieving this pharmacologically. The vascular effects following oral administration of BH_4 appear complex and dose dependent [87, 103]. The ratio of BH_4:BH_2 achieved within vascular endothelial cells may influence outcome and oral BH_4 supplementation could counter-intuitively elicit harmful effects through enhanced prevalence of the BH_2 species [87, 103]. A detailed understanding of both the endogenous regulation of BH_4 in normal physiology and pathophysiology, its cellular uptake/recycling and the fate of orally administered BH_4, appears necessary in order to develop safe, rational BH_4 modifying strategies for the treatment of endothelial dysfunction [117].

Treatment	Cardiovascular effect	Species	Reference
BH_4	augmented relaxation induced by acetylcholine in diabetic aortic rings	Rats	82
BH_4	restoration of eNOS function and reduced vascular oxidative stress in insulin-resistant rats	Rats	83
BH_4	Improves endothelial function and prevents the development of hypertension in SHR	Rats	84
Sepiapterin	improved endothelium-dependent vasodilatation in aortas from $apoE^{-/-}$ mice	Mice	85
BH_4	Improved contractile and metabolic abnormalities in reperfused hearts	Rats	86
BH_4	Improves endothelial dysfunction and vascular oxidative stress in microvessels of intrauterine undernourished rats	Rats	87
BH_4	Prevented the development of hypertension and myocardial hypertrophy associated with long-term infusion of Ang II	Rats	88
Sepiapterin	Improved vascular relaxation in persistent pulmonary hypertension of newborn only when co-administered with superoxide dismutase (MnTMPyP)	Pig	77
BH_4	Protection in myocardial ischaemia	Rats	81
BH_4	Improved endothelial dysfunction, attenuates increased NADPH oxidase mRNA and inflammatory factors in the aortas of $ApoE^{-/-}$mice	Mice	80
BH_4	Inhibits hypoxic pulmonary vasoconstriction	Rats	79
BH_4	Reduced atherosclerosis and in $ApoE^{-/-}$mice	Mice	89
BH_4	Protection following pressure-overload induced hypertrophy. Limited dose range	Mice	90

Table 1 Effects of pharmacological BH_4 administration in animal studies

Disease	Administration	Patient number	Outcome	Reference
Hypercholesterolemia	6R-BH_4 (Alexis Corp.) 10, 100, 500, 1000 μg/min for 5 min	13	Restores endothelial dysfunction	91
Diabetes (type II)	6R-BH_4 (Schricks) intra-arterial infusion 500 μg/min.	23	Improves endothelium dependent vasodilatation	75
Chronic smoking	6R-BH_4 (Schricks) 500 μg/min	16	Improves endothelial function	102
Coronary heart disease	6R-BH_4 (Alexis Corp.) intracoronary infusion 10^{-2} M/min for 2 min	19	Prevents endothelial dysfunction	92
Hypertension	6R-BH_4 (Sigma) 500 mg/min	16	Augments endothelium dependent vasodilatation in normotensive and hypertensivespatients	93
Hypercholesterolemia	6R-BH_4 (Sigma) Intracoronary 1mg/min	18	Improves coronary endothelial function	74
Vasospastic angina (VA)	6R-BH_4 (Sigma) Intracoronary 1mg/min; 2 min	28	Improves coronary endothelial function but does not prevent coronary spasm in VA patients	94
Coronary artery disease	6R-BH_4 4 mg/min	15	Improves endothelium-dependent vasodilatation	78
Diabetes (type II)	6R-BH_4 (Schricks) intra-arterial infusion 500 μg/min	32	Increases insulin sensitivity but does not improve endothelial function	101
Hypercholesterolemia	6R-BH_4 (Schricks) 10 mg/kg 30 min i.v. infusion	19	Improves dysfunction of coronary microcirculation in hypercholesterolaemic patients	95
Endotoxin-induced endothelial dysfunction	6R-BH_4 (Cinalfa) 500 μg/min for 47 min	8	Restores impaired endothelial function	96
Vascular aging	6R-BH_4 (Schricks) 10 mg/kg once	31	Improves endothelium dependent dilation	97
Vascular aging	6R-BH_4(Sigma) 500 mg/min	37	Prevents endothelial dysfunction	98
Ischemia reperfusion injury	6S and 6R-BH_4 and NH_4 (Schircks) 500 μg/minH_4	48	Both 6S and 6R BH_4 improve endothelial function	99
Atherosclerosis	6R-BH_4 (Clinalfa) intracoronary infusion 500 μg/min	57	Does not improve endothelial function	100

Table 2. Acute effects of BH_4 supplementation in human cardiovascular disease

Disease	Administration	Patient number	Outcome	Reference
Chronic smoking	2 mg/kg Sapropterin hydrochloride (single oral dose, measurements taken 3 and 24hr after admin) Biopten.	17	Improved vascular NO bioactivity-increased endothelium-dependent vasodilatation	110
Hypertension	1, 5 or 10 mg kg /day of BH_4 for 8 wks Schircks laboratory (BH_4 preparations compounded with an equivalent amount of Vit C).	8 16	Reduced systolic pressure and MAP and improvement of endothelial function at higher doses	109
Hypercholesterolemia (LDL>4.5 mmol/L)	400 mg of BH_4twice daily over 4 wks. Schircks laboratory	22	Reversal of endothelial dysfunction and oxidative stress	111
Pulmonary arterial hypertension (Safety study)	2.5 mg/kg to 20mg/kg of Sapropterin dihydrochloride over 8 wks. Biomarin	18	Safety study revealed improvement in 6-minute walk distance Efficacy trial ongoing	112
Hypertension	5mg/kg of oral Sapropterin dihydrochloride twice daily for 8 wks. Biomarin	84	No significant effect on systolic blood pressure	NCT00325962 see 108
Endothelial dysfunction	5mg/kg of oral Sapropterin dihydrochloride twice daily for 2 wks. Biomarin	52	No significant difference	NCT00532844 see 108
Sickle cell disease	Sapropterin dihydrochloride twice daily, dose escalation (every 4 weeks) 2.5, 5, 10 mg/kg/day, and 20 mg/kg/day for 16 wks. Biomarin	40	Improvement in endothelial function	NCT00445978 see 108
Peripheral arterial disease	400 mg of oral Sapropterin dihydrochloride twice daily over 24 weeks. Biomarin	190	No significant difference	NCT00403494 see 108
Endothelial dysfunction Estrogen-deficiency	BH_4 10mg/kg (single oral dose, measurements taken 3 hrs after admin) Schircks laboratory	33	Increased endothelial dependent vasodilatation in post menopausal women	113
Coronary artery disease	400 or 700mg/day of Sapropterin dihydrochloride for 2-6 weeks. Biomarin	49	No significant difference	106

Table 3. Oral BH_4 supplementation in human cardiovascular disease

In contrast, strategies that attenuate the pathophysiological rise observed in BH_4 under proinflammatory conditions, may be of benefit in the management of conditions such as pain and septic shock [118-122]. Such conditions are underpinned by enhanced and sustained over production of NO derived from newly transcribed inducible NOS (iNOS) or, in the case of pain, additional increases in neurotransmitters such as noradrenaline, generated by AAAH. Understandably, caution must be exercised with any treatment strategies that directly limit BH_4 bioavailability on a chronic basis, given the potentially detrimental impact on NO biology or aromatic amino acid metabolism.

It is important to note that a greater level of complexity arises with strategies that limit BH_4 bioavailability for NOS. Whilst limiting BH_4 may attenuate the extent of an inflammatory response, through minimising iNOS dimerisation and activity in vascular smooth muscle, it would be important to ensure adequate BH_4 bioavailability for eNOS at the level of the microcirculation. Microvascular blood flow has been shown to be compromised in patients with sepsis[123] and therapeutic strategies that improve microvascular perfusion have correlated with reduced multiple organ failure in septic patients[124]. In these situations, supplementation of NO or BH_4 have been suggested to be a method to improve microcirculatory function in sepsis[125, 126]. Clearly the timing of intervention is critical and a more complete understanding of the BH_4 and NO profiles in sepsis is required in order to develop timely strategies for the management of the vascular alterations that occur in septic shock patients.

6.1 Other mechanism of action for BH_4

Besides the clear cofactor roles of BH_4 for AAAH, GEMO and NOS, a number of other roles of BH_4 have been identified. It is clear that BH_4 can behave as a direct antioxidant reacting with both superoxide [127] and more readily with peroxynitrite ($ONOO^-$)[27, 82]. Indeed the rate constant for the reaction between BH_4 and $ONOO^-$ is several fold higher than the reactions for other known antioxidant systems including ascorbate, glutathione or thiol groups, and likely explains the primary antioxidant mechanism of BH_4 [27].

BH_4 can react with molecular oxygen leading to the production of hydrogen peroxide which has been suggested to be an endothelium derived hyperpolarisation factor (EDHF) [128, 129]. Supplementation of BH_4 to iliac arteries modulates arterial function by reducing oxidant stress and enhancing gap junctional communication and electrotonic signalling mediated by EDHF, independent of NO [130].

7. GCH1 manipulation and BH_4 regulation

One possible method of circumventing the potential problems associated with direct oral BH_4 administration would be to regulate BH_4 bioavailability intracellularly, by pharmacologically targeting enzymes involved in BH_4 biosynthesis or recycling. GCH1 performs the first and, under most circumstances, rate limiting step in BH_4 biosynthesis. Thus, small molecules that enhance GCH1 activity would increase BH_4 levels and eNOS derived NO within vascular cells for the treatment of endothelial dysfunction; whilst strategies that attenuate the inflammation induced rise in GCH1 activity could limit the bioavailable BH_4 for iNOS, as a means to reduce pathophysiological inflammation. The remainder of this article will focus on the evidence that supports the pharmacological modulation of GCH1 as a tractable target for both enhancing and inhibiting BH_4 bioavailability.

7.1 Genetic modification of GCH1

The availability of genetically modified mice has greatly contributed to our understanding of BH_4 biology in the cardiovascular system. Ubiquitous knockout of GCH1 is lethal *in utero* but inducible or conditional knockout of GCH1 may be feasible. Reduced GCH1, activity as observed in the Hph-1 mouse (generated by chemical mutagenesis) [131], results in reduced BH_4 levels, NOS activity and mice exhibit a pulmonary hypertensive phenotype [73, 132]. Conversely, endothelial cell targeted over

expression of GCH1 in mice elevates BH_4 levels, NOS activity and NO levels and mice display resistance to DOCA-salt induced systemic hypertension, hypoxia induced pulmonary hypertension and exhibit reduced atherosclerosis and endothelial dysfunction when crossed with Apo E mice [73, 133, 134]. Following transluminal wire injury, GCH1 over expressing mice also display reduced neointima formation [135] . Similarly adenoviral GCH1 gene transfer into endothelial cells enhances NO production and eNOS dimerisation and improves vascular function [136] [137] whilst siRNA induced knockdown elevates blood pressure in mice [138].

There are also a few, albeit small scale, studies in humans indicating that GCH1 polymorphisms result in a loss of function phenotype with concomitant detrimental effects on vascular function [139-142]. In summary, loss of function of GCH1 results in predisposition to cardiovascular disease whilst over expression appears protective. These effects of experimental gene manipulation or existing human polymorphisms appear to be related to BH_4 bioavailability and NOS functionality.

7.2 Physiological and pharmacological modifiers of GCH1

There are a number of agents that can stimulate BH_4 production, many of which achieve this via increasing the expression or activity of GCH1. Up regulation of GCH1 has been reported at the level of transcription and translation by hormones including insulin [143] and oestrogen [144], physiological stimuli such as shear stress [145] and pharmacological agents including statins [146]. These agents are known to elicit vascular protective effects and may thus achieve this, in part, through enhanced BH_4 bioavailability for NOS. Indeed, these studies support the observed protective effects of BH_4 supplementation in situations where such endogenous mechanisms have become dysfunctional [72, 110]

Similarly in a pathological setting, proinflammatory cytokines have been shown to upregulate GCH1 and BH_4 levels [147-149], whilst anti-inflammatory cytokines such as IL-4 and IL-10 and glucocorticoids have been shown to reverse this rise in BH_4 [150] [151]. Proinflammatory cytokines are known to contribute to an inflammatory response by transcribing iNOS and enhancing reactive oxygen species generation and the generation of micromolar levels of NO, which have both protective antimicrobial and deleterious inflammatory effects. Indeed, aberrant iNOS mediated vascular NO production is also known to acutely contribute to the circulatory collapse observed in septic shock, and studies have shown that inhibition of GCH1 or antagonism of BH_4 binding to NOS, elicits protective effects in animal models of sepsis [119, 120, 152].

8.0 Pharmacological enhancers and inhibitors of BH_4 bioavailability

As described in section 4.0, a stable preparation of BH_4 (sapropterin dihydrochloride) has been granted FDA and EMEA approval as a means to regulate blood phenylalanine levels in a subset of hyperphenylalaninaemic patients. This compound is currently undergoing clinical investigation in a number of cardiovascular diseases although, unfortunately, initial results suggest sapropterin dihydrochloride lacks efficacy (Table 3).

Sepiapterin, a precursor which is converted to BH_4 via the salvage pathway (dihydrofolate reductase) (Fig. 1), is used experimentally to enhance BH_4 levels and has been shown to elicit a restorative effect of endothelial function in a number of cardiovascular disease models [74, 82, 153]. However at high concentrations, sepiapterin may lead to an accumulation of 7,8-BH_2 which in turn may lead to NOS uncoupling and paradoxically elicit detrimental effects [154].

The B vitamin, folic acid has also been shown to elicit beneficial cardiovascular effects by both lowering homocysteine levels and through antioxidant effects which are likely mediated by its circulating reduced biologically active form, 5-methyltetrahydrofolate (5-MTHF). 5-MTHF administration restores BH_4 bioavailability, eNOS activity and endothelial function in human tissue [155, 156]. These studies provide further evidence that the oxidative inactivation of BH_4 underlies numerous cardiovascular pathologies and that strategies limiting BH_4 oxidation, hold therapeutic promise. Finally, stable compounds that can directly activate NOS via the pterin binding site, have been developed. A stable analogue of BH_4 (6-acetyl-7,7-dimethyl-7,8-dihydropterin or ADDP) has been shown to substitute for BH_4 when the pterin binding site on NOS is unoccupied, eliciting beneficial effects on pulmonary vascular function [157, 158].

Pharmacological inhibitors which block the BH_4 biosynthetic or recycling pathways include the prototypic GCH1 inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP) [83, 119, 120, 159-161], and guanine mimetics including 8-azaguanine and 8-mercaptoguanine [162]; methotrexate, an inhibitor dihydrofolate reductase [30, 116]; and N-acetyl-serotonin which can inhibit sepiapterin reductase activity [163-165].

Interestingly, some reagents that reduce BH_4 bioavailability or BH_4 binding to NOS, have demonstrated selectivity for iNOS functionality/expression [164, 166]. Indeed, of the three NOS isoforms, iNOS dimer stability is especially sensitive to BH_4 levels [167]. Thus small molecules that attenuate the

cytokine induced rise in BH_4 (normally necessary to support the stabilisation and activity of newly formed iNOS) may selectively inhibit iNOS function, potentially by destabilising/preventing dimer formation, whilst sparing eNOS mediated NO production.

9.0 GCH1 protein interactions

A number of GCH1 interacting proteins have been identified through yeast two hybrid and immunoprecipitation assays in tissues, some of which appear to be organ specific interactions [7, 168]. Certain proteins such as tyrosine hydroxylase in drosophila [169], Heat shock protein 90 (Hsp90) related proteins and GCH1 feedback regulatory protein (discussed later) [7, 168] can be intuitively linked to the role of GCH1 and BH_4 in AAAH and NOS functionality. Hsp90 is an essential component of a chaperone complex that associates with eNOS and has been shown to be a dynamic regulator of eNOS activity in endothelial cells [170]. Interestingly GCH1 associates with the activator of Heat Shock 90 kDa Protein (Aha1) suggesting that this may be a mechanism whereby GCH1 may be recruited into endothelial cells to regulate BH_4 bioavailability for eNOS [7]. Furthermore, the functional significance of the associations between GCH1, Hsp90 and the C-terminus of Hsp70-interacting protein (CHIP), have recently been demonstrated in a model of congenital heart disease and increased pulmonary blood flow (shunt lambs) where NO signalling is diminished in the pulmonary vasculature [171].

Other interacting proteins such as rabphilin-3A [172], the mitochondrial protein very long-chain specific acyl-CoA dehydrogenase (VLCAD) and eukaryotic translation initiation factors including EIF3I indicate that GCH1 may regulate other physiological processes although these remain to be demonstrated in functional systems [168].

10.0 Post translational modification of GCH1

In addition to the pharmacological and physiological GCH1 regulators, GCH1 activity has also been shown to be controlled post translationally by phosphorylation [145, 173, 174] and via a functional protein-protein interaction [175].

10.1 GCH1 Phosphorylation

Protein phosphorylation has been shown to modulate GCH1 resulting in elevated activity and an increase in cellular BH_4 levels. Examination of the amino acid sequence of GCH1 reveals several conserved putative phosphorylation motifs for casein kinase II and protein kinase C.

Agents such as angiotensin II and platelet derived growth factor (PDGF-BB) have been shown to stimulate GCH1 phosphorylation in a protein kinase C dependent manner [174]. Of the numerous putative phosphorylation motifs on GCH1, serine 81 (within the human sequence) has been shown to be functionally important and regulated by laminar shear stress [145]. These data suggest that BH_4 bioavailability may be dynamically regulated endogenously via the phosphorylation of GCH1. It still remains to be determined whether the other GCH1 phosphorylation motifs are functionally important.

10.2 GCH1 feedback regulatory protein

In addition to phosphorylation, biochemical studies have demonstrated that GCH1 is also subject to allosteric feedback inhibition by BH_4 and feed forward activation by the essential amino acid L-phenylalanine (L-Phe). However, this effect is only seen when GCH1 is bound to GCH1 Feedback Regulatory Protein (GFRP) [175].

A series of elegant studies by Maita *et al*., solved the crystal structures of the rodent GCH1–GFRP complex (residues 48-241 of GCH1 and residues 1-84 of GFRP) identifying the binding sites and interacting residues for both BH_4 (BH_2 was used experimentally due to increased stability) and L-Phe binding within the GCH1–GFRP complex [176, 177]. Crystallisation of the complete GCH1 protein was not possible due to proteolytic cleavage at arginine residues occurring within the first 47 amino acids of the N-terminus. Whilst it was suggested that this N-terminal portion was unlikely to be involved in substrate binding or catalysis [178], other studies have indicated that this N-terminal portion may indeed associate with GFRP and may further elicit regulatory effects on enzyme activity [7, 179].

10.3 BH_4 mediated inhibition and L-Phe mediated activation of the GCH1–GFRP complex

When present in excess, ten molecules of BH_4 bind to the GCH1–GFRP complex, within binding pockets that are exclusively located on GCH1 [176]. This binding induces conformational changes within the active site (in particular residues Phe-113 and Glu-115) potentially leading to incorrect positioning of the substrate GTP, reducing the Vmax of the enzyme and thus reducing GCH1 activity.

Conversely, inhibition of GCH1 activity can be reversed by ten molecules of L-Phe, which bind exclusively within pockets located on GFRP and can stimulate enzyme activity in the presence of sub saturating GTP concentrations [177]. Thus L-Phe changes the protein complex conformation back to an active state and enhances BH_4 biosynthesis [162, 175, 177, 180]. Biopterin or L-Phe binding appear to enhance the association of GFRP with GCH1 by occupying the spaces at the interfaces thereby increasing the surface contact area between the two proteins.

The interaction of GCH1 with GFRP therefore renders GCH1 sensitive to changes in prevailing BH_4 and L-Phe concentrations. Fluctuations in circulating L-Phe, resulting from altered dietary intake, are therefore kept within a physiological range through the feed forward activation of BH_4 biosynthesis which in turn is a necessary cofactor for the hydroxylation reaction mediated by PAH [1, 175]. Similarly, BH_4 levels are kept within an appropriate physiological range through feedback inhibition of GCH1 [175].

Despite advances in our understanding of the physical interactions of GCH1 and GFRP [7, 176, 177, 180], the functional significance of the GCH1–GFRP interactions *in vivo,* has yet to be fully elucidated. However, evidence has demonstrated that these proteins do indeed interact *in vivo*. Firstly, an oral challenge of L-Phe in humans elicits an increase in plasma biopterin (a measurable correlate of plasma BH_4) and a rise in plasma and tissue BH_4 in mice (Starr and Nandi, unpublished), indicating that L-Phe stimulates GCH1 activity in vivo [181, 182]. Secondly, administration of L-Phe increases endothelium-dependent relaxation and attenuates hypertension in rats made hypertensive by GCH1 inhibition [183]. Finally, human epidermal melanocytes and keratinocytes have the capacity to regulate *de novo* BH_4 synthesis via a GCH1–GFRP axis, suggesting that the same regulatory axis likely exists in other cell types which co-express these two proteins [184].

Post translational regulation of GFRP has also been reported *in vitro*. Whilst proinflammatory cytokines up regulate GCH1, BH_4 levels and iNOS activity, a concurrent down regulation of GFRP expression has been reported [185, 186]. It has been postulated that this combination of increased GCH1 and decreased GFRP may render GCH1 insensitive to feedback inhibition by the generated BH_4. This in turn would provide a continuous supply of BH_4 for the newly formed iNOS sustaining a high NO output. In contrast, under healthy naïve conditions, cellular BH_4 levels are likely to exist in sufficiently high concentrations and be tightly bound to eNOS. Thus, any changes in GFRP expression would be unlikely to alter eNOS functionality.

In support of this, we and others have previously demonstrated that over expression of GFRP in endothelial cells did not affect basal BH_4 and eNOS mediated NO production [187, 188] but following proinflammatory stimulation attenuated the rise in BH_4 and consequently dampened iNOS mediated NO production [188]. Therefore the relative ratio of GCH1:GFRP would likely influence the frequency of interaction between the two proteins and hence impact on the extent of feedback inhibition/feed forward activation elicited by BH_4 and L-Phe. This would subsequently determine the amount of bioavailable BH_4 and in turn NOS functionality. Whether the ratio of these two proteins is regulated and altered during pathophysiology, remains to be investigated.

In addition to proinflammatory stimuli, laminar shear stress appears to regulate the GCH1-GFRP complex. Laminar shear – a physical stimulus which is known to elicit vascular protective effects (as opposed to oscillatory shear), has been shown to phosphorylate GCH1 (Ser-81) leading to a significant increase in BH_4 levels [145]. Interestingly, a recent study by the same group demonstrated siRNA mediated knockdown GFRP enhanced GCH1 phosphorylation and that laminar shear caused in a disassociation between GFRP and GCH1 [189]. This data provides further evidence that modification of GCH1-GFRP protein-protein interactions and associations may have the capacity to regulate BH_4 levels in pathophysiology.

10.4 GCH1–GFRP protein-protein interactions – a druggable target?

The pharmacological modulation of the allostery or physical interactions between GFRP–GCH1 offers an interesting therapeutic approach to control BH_4 bioavailability. This target is especially attractive as it provides the potential for both the activation and inhibition of GCH1, and hence the regulation of BH_4 in pathologies where NO is either limited (e.g. endothelial dysfunction) (Figure 2) or where NO contributes to pathophysiology (inflammatory pain and septic shock) (Figure 3).

Two GFRP pentamers form a sandwich like complex surrounding one GCH1 homodecamer [176, 177]. There are 10 identical BH_4 binding sites located on the GCH1 homodecamer and 5 identical L-Phe binding sites located on each GFRP pentamer. Both sets of ligand binding sites reside within the GCH1-GFRP interface [176, 177].

It has been postulated that the phenylalanine-mediated binding of GFRP locks GCH1 into an active conformation such that effective substrate turnover occurs even at low GTP concentrations [175]. Small molecules that can mimic the conformational changes that occur in response to L-Phe binding, therefore have the potential to activate GCH1 and enhance intracellular BH_4 levels. Similarly, those that mimic the changes induced by BH_4 would have the opposite effect in situations where an over production of BH_4 and NO contributes to pathology. However, it has been argued that these binding sites may be poor drug targets because they exist on the interface of two large proteins with a large surface area [175, 190].

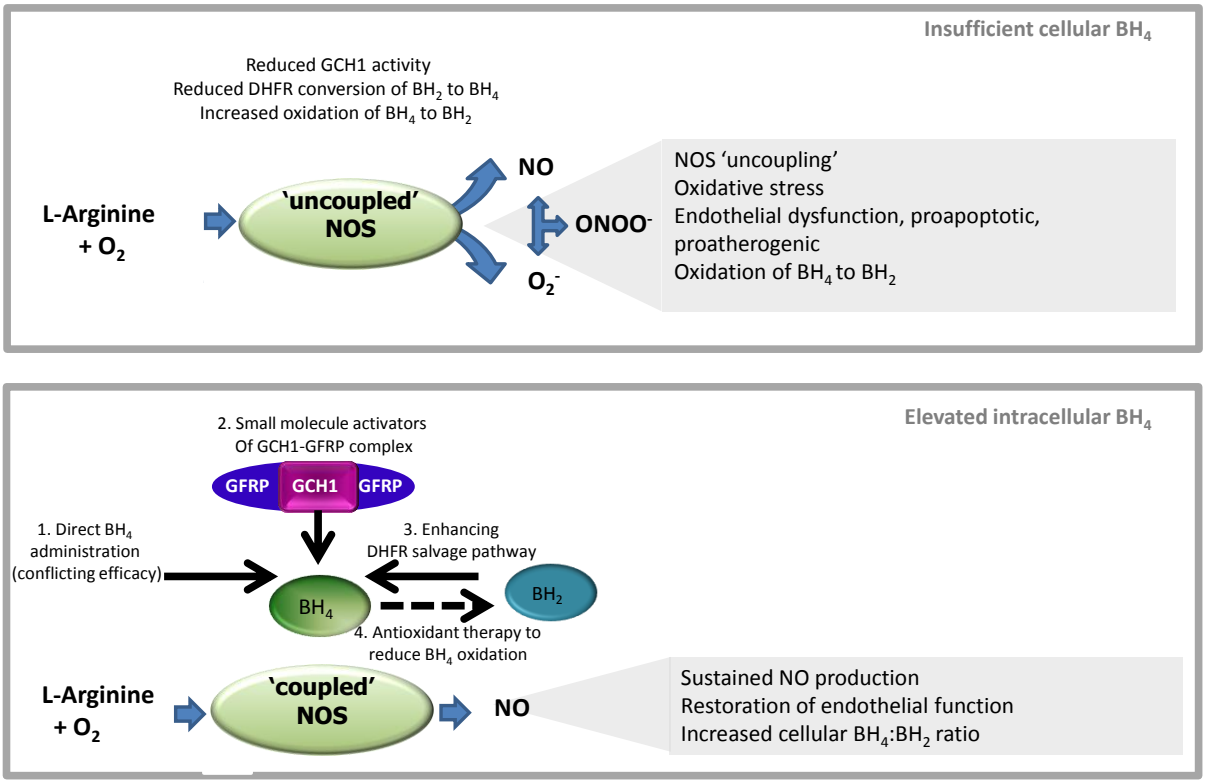

Figure 2: Potential mechanisms to enhance BH_4 levels to treat endothelial dysfunction. Upper panel illustrates effects of insufficient BH_4. Lower panel illustrates methods to enhance BH_4 by 1) direct exogenous BH_4 administration; 2) Small molecule activators of GCH1-GFRP complex; 3) Enhancing DHFR mediated conversion of BH_2 to BH_4 4) antioxidant therapy

Interestingly, advances in the understanding of protein-protein interaction biology [191, 192] have suggested that the presence of energetic 'hot spots' within protein-protein interfaces that may be amendable to pharmacological modification. Thus, a more detailed understanding of the two proteins and structural changes that occur in response to feedback inhibition elicited by BH_4 and feed forward activation elicited by L-Phe, may identify regions other than the known ligand binding sites, which could provide a starting point for rational drug design. Such small molecules could either mimic the changes induced by L-Phe or BH_4 or they could weaken or strengthen the physical interaction between the two proteins [193]. Whether the GCH1–GFRP complex is amenable to this type of drug discovery remains to be determined, but a detailed understanding of the biophysical interactions between the two proteins in the presence of natural ligands, in normal physiology and pathophysiology certainly warrants investigation.

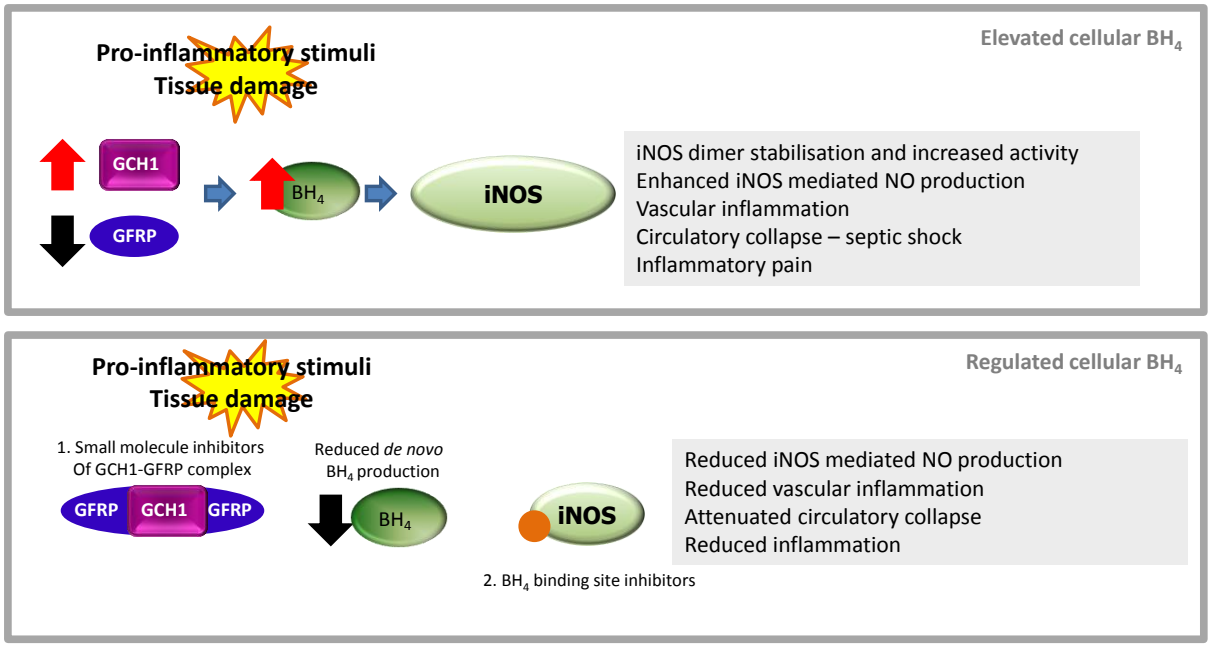

Figure 3. Mechanisms to regulate cellular BH_4 under proinflammatory conditions. Upper panel illustrates proinflammatory stimulation of BH_4 biosynthesis. Lower panel illustrates methods to regulate iNOS mediated NO production under inflammatory conditions: 1) Small molecule inhibitors of GCH1-GFRP; 2) Inhibitors of BH_4 binding to NOS

11.0 Conclusion

Elevating vascular BH_4 levels for the treatment of diseases characterised by endothelial dysfunction holds significant therapeutic potential based on in clinical and preclinical animal model data. Similarly attenuating the rise in BH_4 during inflammatory states could be used to treat the circulatory collapse observed in septic shock patients. Oral administration of BH_4 may lack efficacy possibly as a result of oxidative inactivation during absorption.

Manipulation of BH_4 levels could be achieved at an intracellular level by targeting the activity of enzymes involved in its biosynthesis or recycling. In particular, the rate limiting enzyme, GCH1 can be dynamically regulated (i.e. both activated and inhibited) through allosteric interactions when bound to its regulatory protein, GFRP. This protein-protein complex may offer a novel mechanism to control BH_4 bioavailability at a cellular level for the treatment of a broad spectrum of diseases.

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